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Metabolomics-based component profiling of hard and semi-hard natural cheeses with gas chromatography/time-of-flight-mass spectrometry, and its application to sensory predictive modeling

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Gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) was used to analyze hydrophilic low molecular weight components, including amino acids, fatty acids, amines, organic acids, and saccharides, in cheese, and the sensometric application for practical metabolomic studies in the food industry is described. Derivatization of target analytes was conducted prior to the GC/TOF-MS analysis. Data on 13 cheeses, six Cheddar cheeses, six Gouda cheeses and one Parmigiano-Reggiano cheese, were analyzed by multivariate analysis. The uniqueness of the Parmigiano-Reggiano cheese metabolome was revealed. Principal component analysis (PCA) showed no grouping of the Cheddar cheeses and Gouda cheeses according to production method or country of origin. The PCA loading plot confirms that many amino acids contribute positively to PC1, suggesting that PC1 is closely related to degradation of proteins, and that lactic acid contributed positively to PC2, whereas glycerol contributed negatively to PC2, suggesting that factors regarding degradation of carbohydrates and fats were expressed in PC2. Partial least squares (PLS) regression models were constructed to predict the relationship between the metabolite profile and two sensory attributes, "Rich flavor" and "Sour flavor", which were related to maturation. The compounds that play an important role in constructing each sensory prediction model were identified as 12 amino acids and lactose for "Rich flavor", and 4-aminobutyric acid, ornithine, succinic acid, lactic acid, proline and lactose for "Sour flavor". The present study revealed that metabolomics-based component profiling, focusing on hydrophilic low molecular weight components, was able to predict the sensory characteristics related to ripening.

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[**Key words:** Natural cheese; Metabolomics; GC/TOF-MS; Descriptive sensory analysis; Sensory predictive modeling]

Cheese is a highly desirable dairy product appreciated by consumers all over the world. Cheese is divided broadly into two categories, natural cheese and processed cheese. Natural cheese is a fermented milk-based food product, and differs depending on the area in which it is produced, the specific lactic acid bacteria used as starter, the method of making the cheese, and the ripening time. Natural cheese is made directly from milk, whereas processed cheese is a cheese-based food produced by comminuting, melting and emulsifying one or more natural cheeses and optional ingredients into a smooth homogeneous molten blend using heat, mechanical shear and (usually) emulsifying salts (1). In Japan, approximately 40% of the cheese consumed is processed cheese; therefore, natural cheese is important not only because it is directly consumed, but also because it is the primary ingredient for processed cheese.

Natural cheese flavor is one of the most crucial criteria impacting both consumer acceptance and the quality of processed cheese. Traditional tools for the sensory evaluation of cheese quality include grading and judging. These methods work well when a large number of samples need to be rapidly assessed for basic quality, and they continue to be used in the cheese industry for this purpose. Cheese grading and judging results, however, do not necessarily reflect consumer preferences, and are thus not ideal tools for cheese flavor research. Currently, the most powerful sensory tool in cheese flavor research is descriptive sensory analysis (2).

Several studies on cheese flavor characteristics have been conducted using descriptive sensory analysis (3–7). Since precise sensory evaluation is both time-consuming and expensive, many studies have focused on particular compounds that affect specific sensory characteristics. These studies were aimed at substituting time-consuming and costly sensory evaluation with instrumental analysis. Many studies concerning the odor-active volatiles in cheese have been performed, resulting in the identification and quantification of the volatile compounds, for example, responsible for the nutty flavor in Cheddar cheese (8), and the characterization of the aroma of Gouda-type cheeses (9). Recent studies have focused on the hydrophilic taste-active compounds in cheese, such as the water-soluble extracts in Cheddar cheese (10), Gouda cheese (11), Swiss

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cheese (12,13), Comte cheese (14) and goat's milk cheese (15). However, the complexity of cheese makes it difficult to apply a purely scientific approach to the cheese industry; therefore, artisans' skills, based on years of experience, are still required for quality maintenance during the cheese-making process.

Metabolomics is an effective post-genome research tool that has been applied to many disciplines, including the study of human disease, nutrition, drug discovery, and plant physiology. In food science, metabolomics has recently been used to monitor the quality, processing and safety of both raw materials and final products (16). Various separation and detection techniques have been used, such as nuclear magnetic resonance (NMR), gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and capillary electrophoresis/mass spectrometry (CE/MS). GC/MS provides high resolution and reproducibility, and is frequently used to identify compounds through the analysis of fragmentation patterns. Innovative techniques have been developed to analyze metabolic fingerprints using GC/MS; for example, to assess the quality of the Chinese medicine *Angelica acutiloba* (or Yamato-toki) roots according to cultivar and species (17), and to predict green tea sensory quality (18).

Natural cheese is made from cow's milk by first adding rennet and lactic acid bacteria starter. The final cheese is obtained after a multi-step manufacturing process. Biochemical changes occur in the cheese, especially during ripening, resulting in the development of a specific flavor, aroma and texture (19). A number of factors, including the composition of the ingredients (added salts, enzymes and lactic acid bacteria), and the degradation and catabolism that occur during ripening, result in considerable diversity in compounds that lead to the phenotype expressed as 'cheese quality'. Since it is difficult to manage cheese quality using a conventional approach focused on one or several target compounds, metabolomics is a good tool for this application.

The application of metabolomics represents a major advance towards understanding cheese quality in terms of the components of the metabolome, and would allow application of this understanding to ensure quality, as well as demystify cheese making in general. However, there is no published research concerning metabolome analysis with GC/TOF-MS targeting hydrophilic low molecular weight compounds in natural cheeses, nor of the application of metabolome analysis to sensometric applications.

This study focused mainly on Cheddar cheese and Gouda cheese, which are the most popular cheeses in Japan. Our goals were to: (i) conduct metabolic profiling of hydrophilic low molecular weight compounds, including amino acids, fatty acids, amines, organic acids, and saccharides, with GC/TOF-MS and multivariate analysis, (ii) construct a sensory predictive model corresponding to descriptive sensory analysis using metabolic profiling, and (iii) identify the groups of components that contribute to the predictive model.

MATERIALS AND METHODS

Natural cheese samples Thirteen natural cheeses [six Cheddar cheeses (1_Ch, 2_Ch, 3_Ch, 4_Ch, 5_Ch, and 6_Ch), six Gouda cheeses (7_Go, 8_Go, 9_Go, 10_Go, 11_Go, and 12_Go) and one Parmigiano Reggiano cheese (13_Pr)] were used in this study. The countries of origin of these samples were Australia (1_Ch and 6_Ch), UK (5_Ch), the Netherlands (7_Go, 11_Go, and 12_Go), Italy (13_Pr), and Japan (2_Ch, 3_Ch, 4_Ch, 8_Go, 9_Go, and 10_Go). These samples were purchased from a local market in Kanagawa Prefecture, Japan (3_Ch, 4_Ch, 8_Go, 9_Go, and 10_Go) or directly from the manufacturers (1_Ch, 2_Ch, 5_Ch, 6_Ch, 7_Go, 11_Go, 12_Go, and 13_Pr) .The dates of manufacture of these samples were for 1_Ch in May 2009, 2_Ch in November 2009, 5_Ch in April 2008, 6_Ch in February 2008, 7_Go in January 2009, 8_Go in August 2009, 11_Go in November 2007, 12_Go in August 2009, and 13_Pr in February 2008. 3_Ch, 4_Ch, 9_Go, and 10_Go were domestic products sold at a Japanese market and only had a "best before" date: 3_Ch in May 2010, 4_Ch in March 2010, 9_Go in March 2010, and 10_Go in February 2010.

Sample preparation (extraction and derivatization) for instrumental analysis was conducted at approximately the same time as sensory evaluation. Samples were stored at 4°C until used for sensory evaluation, and subsequently stored at −20°C until used for sample preparation.

Reagents All chemicals used in this study were of analytical grade. Methanol and chloroform were used as extraction solvents, ribitol was used as an internal standard, and pyridine was used as a solvent (Wako, Osaka, Japan). Methoxyamine hydrochloride was purchased from Sigma (St. Louis, MO, USA). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide was purchased from GL Sciences, Inc. (Tokyo, Japan).

Sample preparation of natural cheeses for GC/TOF-MS analysis Cheese samples were frozen in liquid nitrogen, ground, and then freeze-dried. Freeze-dried cheese (100 mg) in 2-mL Eppendorf tubes was extracted with 1000 μL $MeOH/H_2O/CHCl_3$ (2.5/1/1, v/v/v). Sixty microliters of 0.2 mg/mL ribitol, used as an internal standard, was added to the mixture, mixed with 5-mm diameter zirconia beads using a vortex mixer, suspended in a ball-mill (20 Hz, 1 min, room temperature) and then sonicated (1.5 min × 3).

The sample was centrifuged at 16,000 × *g*, for 3 min at 4°C, and then 800 μL of the supernatant was transferred to a 1.5-mL Eppendorf tube. Water (400 μL), purified using a Millipore Milli-Q system (Bedford, MA, USA), was added and the sample was vortexed. Following centrifugation (16,000 × *g*, 3 min at 4°C), 500 μL of supernatant was transferred to another 1.5-mL Eppendorf tube and capped. The cap was subsequently pierced and the extract was evaporated, to remove methanol, in a centrifuge vacuum concentrator at room temperature for approximately 2 h. After evaporation, the extract was freeze-dried in a glass bottle at room temperature overnight.

Sample derivatization For derivatization, 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to the above freeze-dried sample, and the mixture was incubated in a Thermomixer comfort (Eppendorf, Tokyo, Japan) at 30°C for 90 min to induce the methoxyation reaction. A second derivatizing agent, 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, was added and the mixture was incubated at 37°C for 90 min to induce the silylation reaction. The samples were then used for GC/TOF-MS analysis. One microliter of sample was injected in split mode (25:1, v/v).

GC/TOF-MS analysis The gas chromatograph used in this study was a 6890N network GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 0.25 mm i.d. fused silica capillary column coated with a 0.25-μm film of CP-SIL 8 CB (Varian Inc., Harbor City, CA, USA). The GC was coupled with a Pegasus III time-of-flight mass spectrometer (LECO Co., St. Joseph, MI, USA) and a 7683B series injector (Agilent Technologies) autosampler. The injection temperature was 230°C. The carrier gas (helium) flow rate through the column was 1 mL/min. The column temperature was held at 80°C for 2 min isothermally and then raised by 15°C/min to 330°C and held for 6 min. The transfer line and the ion source temperatures were 250°C and 200°C, respectively. Ions were generated at 70 eV with electron ionization and were recorded at 20 spectra per second over the mass range *m/z* 85–500, with the detector voltage at 1550 V.

Data processing Raw chromatographic data acquired from GC/TOF-MS (Pegasus file, *.peg) were processed by ChromaTOF (ver. 2.32, LECO), in which automatic peak detection, mass spectrum deconvolution and baseline correction were performed, followed by conversion into an AIA file (ANDI files: Analytical Data Interchange protocol, *.cdf). The ANDI format allowed conversion and transfer of data between different mass spectrum data systems. Peak retention times were aligned and the peak intensities were normalized to the ribitol peak included as the internal standard. Finally, chromatographic data at elution times lacking peaks were deleted. Data treated as above were obtained as the value of intensities at each retention time of each sample.

Metabolite identification Significant compounds were identified by comparing their mass spectra with those in libraries (the NIST library and an in-house library prepared from authentic standard chemicals).

Peak selection The purpose of the present study was to better understand flavor-related compounds. GC/TOF-MS has a wide dynamic range, and if too small peaks had been used for constructing the sensory predictive model, compounds not having any practical influence on sensory properties would have been identified as compounds contributing to model construction. While peak intensity is not always proportional to the amount of a compound, since different compounds might give rise to different intensities in the MS and be derivatized with different efficiencies, it reflects the quantity of a constituent considerably. Therefore, the observed peaks were screened and narrowed down based on a criterion of peak intensity reflecting, to some extent, taste-activity as follows.

According to the literature (11), among the water soluble taste-active compounds (for target analytes in this study) in Gouda cheese, L-lysine (classified into the bitter-tasting amino acids) had the minimum taste activity and a sensory threshold value of 80,000 μmol/kg, and L-aspartic acid (also classified into the bitter tasting amino acid) had the maximum taste activity and a sensory threshold value of 600 μmol/kg. Furthermore, L-lactic acid, exhibiting the maximum peak intensity in the present study, had a sensory threshold value of 23,770 μmol/kg for umami and 11,890 μmol/kg for sour/salty, respectively, as sodium lactate. Making a tentative calculation, the taste activity of L-lysine was approximately 0.3 times (23,770/80,000) that of L-lactic acid, and L-aspartic acid was approximately 40 times (23,770/600) that of L-lactic acid. In essence, the taste activity of L-lactic acid was intermediary compared to the other identified compounds (mainly amino acids) that ranged from 0.3 to 40 times that of L-lactic acid taste activity. Therefore, we included all compounds with peak intensities greater than 1% of that of L-lactic acid. In view of the taste activity for water-soluble taste-active compounds, setting the criteria above would enable us to adopt compounds to be taken up for discussion and prevent the discarding of contributing compounds. As a result, the peaks of 36 compounds (including 10 unidentified compounds) remained for further analysis.

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